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Occurrence of Plasmid- and Chromosome-Carried *mcr-1* in Waterborne *Enterobacteriaceae* in China

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ABSTRACT The aim of this study was to investigate the prevalence of the polymyxin resistance gene *mcr-1* in *Enterobacteriaceae* from environmental water sources in Hangzhou, China. Colistin-resistant bacteria were isolated from environmental water samples using an enrichment broth culture method, were screened for *mcr-1*, and then were analyzed for the location and transferability of *mcr-1*. Isolates positive for *mcr-1* were further examined to determine their susceptibility profiles and were screened for the presence of additional resistance genes. Twenty-three *mcr-1*-positive isolates (16 *Escherichia coli*, two *Citrobacter freundii*, two *Klebsiella oxytoca*, two *Citrobacter braakii*, and one *Enterobacter cloacae*) were isolated from 7/9 sampling locations; of those, eight *mcr-1*-positive isolates also contained β-lactamase-resistance genes, eight contained *qnrS*, and 10 contained *oqx*. No *mcr-2*-positive isolates were identified. The majority of isolates demonstrated a low to moderate level of colistin resistance. Transconjugation was successfully conducted from 14 of the 23 *mcr-1*-positive isolates, and *mcr-1* was identified on plasmids ranging from 60 to 220 kb in these isolates. Conjugation and hybridization experiments revealed that *mcr-1* was chromosome-borne in only three isolates. Pulsed-field gel electrophoresis showed that the majority of *E. coli* isolates belonged to different clonal lineages. Multilocus sequence typing analysis revealed that sequence type 10 (ST10) was the most prevalent, followed by ST181 and ST206. This study demonstrates the utility of enrichment broth culture for identifying environmental *mcr-1*-positive isolates. Furthermore, it highlights the importance of responsible agriculture and clinical use of polymyxins to prevent further widespread dissemination of polymyxin-resistant pathogens.

KEYWORDS chromosome-carried, plasmid-carried, polymyxin resistance, *mcr-1*, *Enterobacteriaceae*, enrichment broth culture

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Carbapenems are the most effective antibiotics for treating infections caused by multidrug-resistant *Enterobacteriaceae*. However, the prevalence of carbapenem-resistant *Enterobacteriaceae* (CRE) is increasing due to the emergence of resistance mechanisms, including the production of subgroup 2f carbapenemases (e.g., *Klebsiella pneumoniae* carbapenemase), metallo-β-lactamases (e.g., Verona integron-encoded metallo-β-lactamase and New Delhi metallo-β-lactamase-1), and extended-spectrum β-lactamases (combined with porin loss) (1–5). In China, the proportion of carbapenem-resistant clinical *Klebsiella pneumoniae* isolates can be as high as 13.4% (6), making it difficult to treat serious infections caused by these multidrug-resistant pathogens. Colistin (polymyxin E), a conventional antibiotic with relatively strong nephrotoxicity,

has therefore attracted much attention as the treatment of last resort for CRE. However, colistin resistance is now frequently observed in *Enterobacteriaceae* (7), and has even been reported in CRE isolates (8).

Resistance to colistin is usually attributed to the chromosome-encoded PmrA/PmrB and PhoP/PhoQ two-component regulatory systems (9). Since it was first reported in foodborne *Enterobacteriaceae* in 2016 (7), the mobile colistin resistance gene *mcr-1* has been found in *Enterobacteriaceae* from animals in South Africa (10), Egypt (11), Vietnam (12), Belgium (13), and Germany (14). It has also been identified in pathogens isolated from the human gut in Latin America (15), Switzerland (16), and China (7). We also identified a high frequency of *mcr-1* in *Escherichia coli* and *Citrobacter freundii* isolated from the stools of children in China (17). In addition, a recent report has indicated that the usually plasmid-borne colistin resistance gene *mcr-1* could also be chromosome carried (18).

Shen et al. (19) conducted a retrospective study in an attempt to identify *mcr-1* in 1,611 *E. coli* isolates collected from chickens in China since the 1970s. They found that *mcr-1* started to emerge as early as the 1980s and that the frequency increased dramatically around 2009, which coincided with the application of polymyxins in agriculture as feed additives. It appears likely that this use of polymyxins has directly triggered the emergence of *mcr-1* in animal-borne pathogens, leading to its proliferation in the environment and transfer among different organisms.

We previously compared the resistance gene profiles of pathogens isolated from humans and the environment (20, 21), showing that environmental bacterial strains had similar quinolone resistance profiles as well as plasmid-carried $bla_{CTX\text{-}M}$. This suggested that environmental water sources might serve as a reservoir and mediator for the transfer of resistance genes among pathogens (20, 21).

There have been numerous reports of *mcr-1* in animal and human pathogens, but fewer studies have addressed *mcr-1*-bearing pathogens from environmental water sources. In this study, we established an enrichment method to increase the detection rate of *mcr-1*-carrying bacteria. As a result, we identified multiple *mcr-1*-positive *Enterobacteriaceae* isolates, including *E. coli*, *Enterobacter cloacae*, *Klebsiella oxytoca*, *Citrobacter braakii*, *C. freundii*, and *Klebsiella pneumoniae*, from nine environmental water sites in Hangzhou, China.

RESULTS AND DISCUSSION

Identification of *mcr-1*-positive isolates from aquatic environments. Using current knowledge about methods of identifying bacteria carrying *mcr-1* or *mcr-2*, we developed an enrichment culture method to analyze water samples from nine aquatic environments in Hangzhou. Seven of the nine sampling locations yielded *mcr-1*-positive isolates, but no *mcr-2*-positive isolates were identified. Water from West Lake and the hospital fountain of the Second Affiliated Hospital of Zhejiang University were negative for both *mcr-1* and *mcr-2*. Nearly 70% of the *mcr-1*-positive isolates (16/23) were *E. coli*. Since the discovery of *mcr-1* in foodborne *E. coli* by Liu et al. (7), this plasmid-carried polymyxin resistance gene has been widely studied (10–13, 19). Four reports focus on *mcr-1* in foodborne and animal-derived *E. coli* isolates (10, 12, 13, 19), while three address the prevalence of this gene in *Enterobacteriaceae* from the gut microbiota of children (7, 15, 17). Only one previous study has reported *mcr-1* in waterborne *E. coli* (16). Our data suggest that *mcr-1*-carrying *E. coli* is common in environmental water sources in Hangzhou, China. We also detected *mcr-1* in *Citrobacter freundii* (two isolates), *Klebsiella oxytoca* (two isolates), *Citrobacter braakii* (two isolates), and *Enterobacter cloacae* (one isolate), indicating the presence of *mcr-1* in several species of *Enterobacteriaceae* distributed across the environmental water system in Hangzhou, China.

The rarity of reports on *mcr-1* from environmental water sources may be a consequence of to the screening method used. Instead of screening environmental water samples directly on agar plates, we incorporated several steps to enrich the *mcr-1*-positive bacteria. First, we used a sterile filtration membrane to trap bacteria from a

1-liter water sample. Second, we used an enrichment broth culture consisting of peptone water and only subjected cultures which were PCR-positive for *mcr-1* to further screening assays. Third, colistin-resistant *Enterobacteriaceae* were further selectively proliferated in *Enterobacteriaceae* enrichment broth containing colistin at 2 μg/ml, which is the breakpoint for colistin resistance (http://www.eucast.org/). Finally, we used MacConkey agar plates containing 2 μg/ml of colistin to select the colistin-resistant colonies. These techniques permitted a high rate of identification of *mcr-1*-carrying bacteria. Our findings verify the utility of enrichment broth culture in the isolation of *mcr-1*-carrying *Enterobacteriaceae* from environmental samples.

Distribution of the *mcr-1*-positive isolates. Among the seven sampling locations from which *mcr-1*-positive isolates were retrieved, one (Huajiachi Lake, where five sites were sampled) provided 10 *mcr-1*-positive isolates (four *E. coli* and two each of *C. freundii*, *C. braakii*, and *K. oxytoca*). Other *mcr-1*-positive isolates were from the Xixi Wetland (four *E. coli* isolates), the Jinghang Grand Canal (one *E. coli* isolate and one *E. cloacae* isolate), the Tiesha River (two *E. coli* isolates), the Qiantang River (two *E. coli* isolates), the East River (two *E. coli* isolates), and Nine Creeks (one *E. coli* isolate).

Huajiachi Lake is located on the campus of Zhejiang Agricultural University, and there are several experimental poultry, pig, and cattle farms near the lake boundary. The possible use of polymyxins and other antibiotics in these farms may have resulted in increased transmission of *mcr-1*-harboring *Enterobacteriaceae* into the environment. Colistin has been widely used in food-producing animals, and the agricultural demand for colistin is expected to reach 16,500 tons/year by 2021 (22). *mcr-1*-carrying *E. coli* is commonly identified in livestock and in their feces (7, 12). The use of polymyxins and other antibiotics in these farms may have contributed to the release of *mcr-1*-harboring *Enterobacteriaceae* into the local environment. In addition, although we postulate that the *mcr-1*-carrying bacteria identified in this study originated from livestock farms as a consequence of agricultural polymyxin use, we cannot rule out human feces as a possible source of *mcr-1*-carrying bacteria.

Identification of other resistance determinants. Through further screening of the *mcr-1*-positive isolates for the presence of several other resistance genes, we identified $bla_{\text{CTX-M-15}}$ in three isolates, $bla_{\text{CTX-M-55}}$ in one isolate, and $bla_{\text{TEM-1}}$ in six isolates. *oqxB* was the most frequently identified plasmid-mediated quinolone resistance (PMQR) gene (10/23 isolates), being found either alone or together with *qnrS* and/or *oqxA* (Table 1).

Antibiotic susceptibility profiles. Although colistin was used for screening purposes in the current study, most *mcr-1*-positive isolates showed moderate resistance to colistin, with MIC values ranging from 1 to 32 μg/ml. The overall rates of resistance of all isolates to the 12 antibiotics were moderate to low, with a few exceptions (Table 1). The MICs of gentamicin and amikacin were ~2 to 32 μg/ml, although a few isolates had extremely high MIC values (>256 μg/ml). In addition, $bla_{\text{CTX-M}}$-carrying isolates showed elevated MICs for cephalosporins.

Transconjugation. To evaluate the transferability of the colistin resistance determinants, we conducted transconjugation experiments and S1-pulsed-field gel electrophoresis (PFGE) combined with Southern hybridization assays. Of 23 isolates, 14 (60.9%) were capable of transconjugating with *E. coli* EC600. The MICs of the transconjugants were also determined and compared with those of the original isolates. The transconjugants (designated with a T) had colistin MICs similar to or slightly lower than those of their corresponding original isolates (Table 1). For example, the MICs of HJCL-1, HJCL-3, HJCL-4, HJCL-5, and HJCL-6 were 1, 4, 2, 2, and 2 μg/ml, respectively, while the MICs of their respective transconjugants were 1, 4, 1, 1, and 2 μg/ml, respectively. A similar pattern was also observed for isolates TSR-1 and TSR-2. However, in several cases (XXWL-1, XXWL-2, XXWL-3, ER-1, ER-2, and JHGC-1), the original strains had lower colistin MICs than their respective transconjugants.

In most cases, the differences in colistin MICs between the original isolates and their transconjugants were within 2-fold (within the range of experimental variation). More-

TABLE 1 Distribution of *mcr-1*-positive *Enterobacteriaceae* isolated from aquatic environments and the MICs of commonly used antibiotics for original isolates and their *mcr-1*-positive transconjugants

Aquatic environment	Isolate no.	Organism	MIC (μg/ml)[a]												Other resistance genes
			IPM	GEN	CTX	TZP	SCF	AMK	TIM	CST[d]	FEP	CAZ	CRO	CIP	
East River	ER-1	*E. coli*	0.19	2	0.047	0.75	0.38	4	3	2	0.023	0.094	0.047	0.25	*qnrS*
	ER-2	*E. coli*	0.125	0.75	>256[b]	1	2	2	4	2	6	4	64[b]	0.012	$bla_{CTX-M-15}$
	ER-1-T[c]		0.19	0.75	0.094	2	0.25	2	4	1	0.047	0.38	0.094	0.125	
	ER-2-T		0.19	0.75	32[b]	3	1	2	6	1	1.5	4	48[b]	0.125	
Huajiachi Lake	HJCL-1	*E. coli*	0.19	0.75	0.047	1.5	0.5	3	4	4[b]	0.047	0.19	0.047	0.006	
	HJCL-2	*E. coli*	0.125	1	0.047	2	0.38	3	2	4[b]	0.064	0.094	0.032	0.75	*qnrS*
	HJCL-3	*E. coli*	0.25	>256[b]	0.094	4	2	>256[b]	24	4[b]	0.38	0.125	0.064	>32[b]	*oqxA*, *oqxB*
	HJCL-4	*E. coli*	0.19	48[b]	0.5	12	1.5	>256[b]	24	2	1.5	0.094	0.064	>32[b]	*oqxB*
	HJCL-5	*C. freundii*	0.25	16[b]	0.25	3	1	24	3	2	0.032	0.75	0.38	1	*qnrB*
	HJCL-6	*C. braakii*	0.5	24[b]	0.38	3	1	32	2	2	0.064	0.75	0.5	0.5	*qnrB*
	HJCL-7	*K. oxytoca*	0.25	2	0.025	1.5	1	32	1.5	32[b]	0.023	0.064	0.047	0.75	*qnrS*, *oqxB*
	HJCL-8	*K. oxytoca*	0.75	1.5	0.025	1	1	24	1.5	32[b]	0.016	0.047	0.032	0.75	*qnrS*, *oqxB*
	HJCL-9	*C. braakii*	0.38	8	0.025	6	4	16	32	4[b]	0.125	0.19	0.38	>32[b]	bla_{TEM-1}, *qnrB*, *oqxA*, *oqxB*
	HJCL-10	*C. freundii*	0.25	8	0.125	4	1	8	8	4[b]	0.125	0.25	0.19	>32[b]	bla_{TEM-1}, *qnrB*, *oqxA*, *oqxB*
	HJCL-1-T		0.25	0.75	0.064	3	0.19	2	4	1	0.047	0.38	0.094	0.125	
	HJCL-3-T		0.25	0.75	0.094	2	0.25	2	4	4[b]	0.064	0.38	0.094	0.125	
	HJCL-4-T		0.19	0.75	0.094	3	0.25	3	6	1	0.064	0.38	0.094	0.125	
	HJCL-5-T		0.19	0.75	0.094	2	0.25	2	4	1	0.064	0.38	0.064	0.125	
	HJCL-6-T		0.19	0.75	48[b]	3	1	2	8	2	2	4	48[b]	0.125	
Tiesha River	TSR-1	*E. coli*	0.094	0.19	0.094	4	1	3	6	1	0.064	0.19	0.064	0.047	bla_{TEM-1}, *oqxB*
	TSR-2	*E. coli*	0.125	0.75	0.125	3	0.75	2	8	1	0.064	0.125	0.064	0.047	bla_{TEM-1}, *oqxB*
	TSR-1-T		0.25	0.75	0.094	2	0.25	2	3	1	0.047	0.25	0.064	0.125	
	TSR-2-T		0.19	0.75	0.094	3	0.25	2	3	1	0.047	0.38	0.094	0.125	
Nine Creeks	NC-1	*E. coli*	0.125	1.5	0.094	2	2	6	12	2	0.094	0.19	0.064	0.5	*qnrS*
Qiantang River	QTR-1	*E. coli*	0.19	1.5	0.19	4	0.5	4	4	4[b]	0.125	0.5	0.094	>32[b]	
	QTR-2	*E. coli*	0.25	1.5	2	>256[b]	6	3	16	4[b]	48[b]	24[b]	>256	0.012	*qnrS*
	QTR-2-T		0.094	1.5	0.047	1.5	0.38	4	2	8[b]	0.047	0.094	0.032	0.19	
Xixi Wetland	XXWL-1	*E. coli*	0.125	32[b]	48[b]	0.38	4	3	8	4[b]	6	8	96[b]	>32[b]	$bla_{CTX-M-55}$, bla_{TEM-1}, *oqxB*
	XXWL-2	*E. coli*	0.19	48[b]	128[b]	1	4	3	16	2	6	8	>256[b]	>32[b]	$bla_{CTX-M-15}$
	XXWL-3	*E. coli*	0.25	32[b]	128[b]	1	4	6	6	2	6	8	>256[b]	>32[b]	$bla_{CTX-M-15}$, bla_{TEM-1}, *oqxB*
	XXWL-4	*E. coli*	0.19	1.5	0.047	1.5	0.25	3	4	2	0.032	0.094	0.047	0.25	*qnrS*
	XXWL-1-T		0.19	0.75	0.094	3	0.25	2	4	1	0.047	0.38	0.094	0.19	
	XXWL-2-T		0.25	1	0.094	3	0.25	2	3	1	0.047	0.38	0.094	0.19	
	XXWL-3-T		0.19	0.75	0.094	2	0.25	2	4	1	0.064	0.38	0.094	0.19	
Jinghang Grand Canal	JHGC-1	*E. coli*	0.19	1.5	0.094	2	0.75	3	12	2	0.064	0.19	0.064	0.5	
	JHGC-2	*E. cloacae*	0.25	1.5	0.125	2	0.5	4	1.5	2	0.047	0.125	0.38	0.19	*qnrS*
	JHGC-1-T		0.19	0.75	0.094	3	0.125	2	4	1	0.064	0.25	0.064	0.125	

[a]IPM, imipenem; GEN, gentamicin; CTX, cefotaxime; TZP, piperacillin-tazobactam; SCF, cefoperazone/sulbactam; AMK, amikacin; TIM, ticarcillin-clavulanic acid; CST, colistin; FEP, cefepime; CAZ, ceftazidime; CRO, ceftriaxone; CIP, ciprofloxacin.
[b]Resistance.
[c]T, transconjugant.
[d]MIC of colistin (CST) was determined using the broth microdilution method.

over, even with similar resistance gene profiles, the MIC can differ as a result of a difference between the genetic environments of the parent isolates and the transconjugants. As shown in Table 1, none of the β-lactamase or PMQR genes examined in this study were identified in the *mcr-1*-positive transconjugants, indicating that *mcr-1* is carried on plasmids separate from those carrying other resistance genes and that these other plasmids either were not transferred or were transferred to different recipients during conjugation. Alternatively, any plasmids that carried both *mcr-1* and other resistance determinants were too large to be successfully transferred by conjugation. Interestingly, Li et al. recently reported that there are three different types of *mcr-1*-bearing plasmids. The two smaller plasmids, ~33 to 60 kb in size, carried only *mcr-1*, while the larger 216- to 280-kb plasmid carried both *mcr-1* and other resistance determinants, including bla_{CTX-M}, bla_{CMY}, bla_{TEM}, *fosA*, *qnrS*, *floR*, and *oqxAB*, in various combinations (23).

S1-PFGE hybridization. We conducted S1-PFGE and Southern hybridization analyses of representative transferrable and nontransferable *mcr-1*-carrying isolates. The

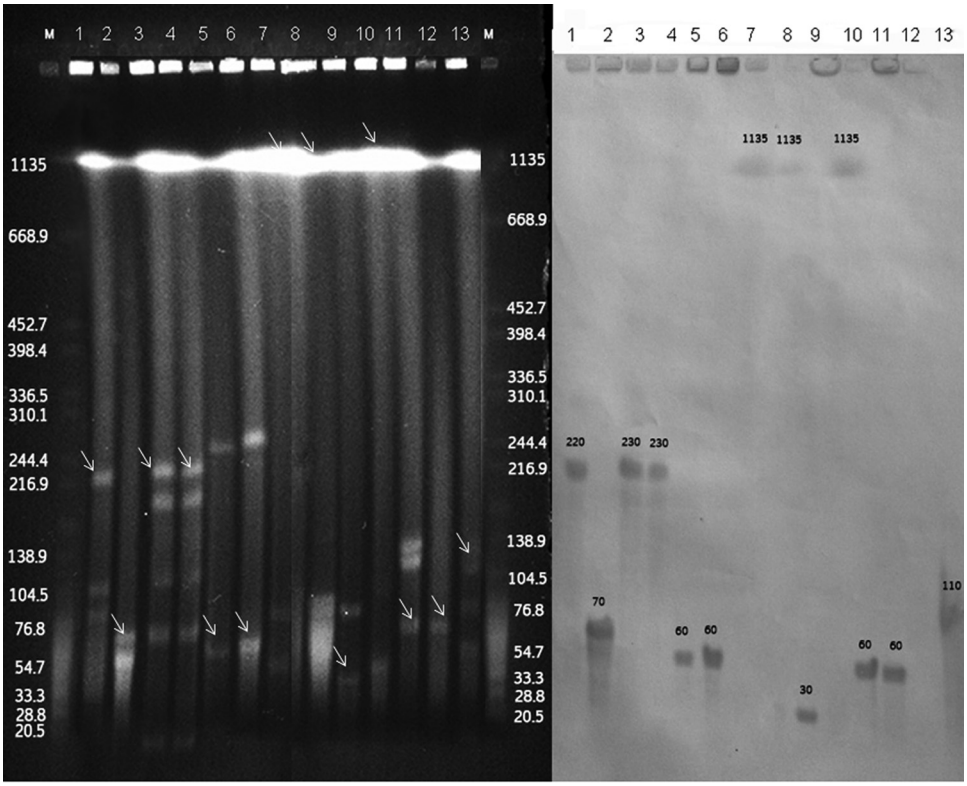

FIG 1 The locations of *mcr-1* from representative isolates, transconjugants, and untransferable isolates were determined by S1-pulsed-field gel electrophoresis (PFGE) and Southern hybridization. Transferable plasmids: lane 1, HJCL-6 (220 kb); lane 2, HJCL-6-J (70 kb); lane 12, HJCL-2 (60 kb); and lane 13, HJCL-2-J (60 kb). Untransferable plasmids: lane 3, HJCL-7 (230 kb); lane 4, HJCL-8 (230 kb); lane 5, HJCL-9 (60 kb); lane 6, HJCL-10 (60 kb); lane 9, QTR-1 (30 kb); and lane 11, JHGC-3 (110 kb). Chromosome: lane 7, HJCL-2; lane 8, NC-1; and lane 10, XXWL-4. The plasmid- and chromosome-carried *mcr-1* loci are marked with white arrows on S1-PFGE fingerprints.

results revealed that the transferable plasmids carrying *mcr-1* (HJCL-6 and HJCL-2) were ~220 kb and ~60 kb, respectively. However, the sizes of nontransferable plasmids (QTR-1, HJCL-7, HJCL-8, HJCL-9, HCJL-10, and JHGC-3) were 30 to 230 kb. In three isolates (HJCL-2, NC-1, and XXWL-4), *mcr-1* was not transferred; in these, the *mcr-1* probe hybridized to a band at ~1,135 kb, the position where the chromosomal DNA was located (Fig. 1). Prior to this study, *mcr-1* was believed rarely to be chromosome borne, although Zurfluh et al. (18) reported one *E. coli* strain containing chromosome-carried *mcr-1*. This supports our finding that a portion of strains contain a chromosomal copy of *mcr-1*.

Molecular typing. We examined the molecular epidemiology of the 16 *mcr-1*-positive *E. coli* isolates using PFGE and multilocus sequence typing (MLST) analyses. Our PFGE analysis indicated that the majority of isolates appeared to be from different clonal lineages, except for the three subclones grouped in Fig. 2. The first subclone consisted of three isolates from the Xixi Wetland (XXWL-1, XXWL-2, and XXWL-3), which were considered to be a single clone based on their identical PFGE patterns and sequence types ([STs] ST206). Moreover, all three isolates carried $bla_{\text{CTX-M}}$ (either 15 or 55) and $bla_{\text{TEM-1}}$. However, another Xixi Wetland isolate, XXWL-4, clustered together with isolate QT-2 from the Qiantang River, and both belonged to ST1638 and carried *qnrS*. Isolates TSR-2 and NC-1 comprised another clonal group and belonged to ST181. However, TSR-2 carried $bla_{\text{TEM-1}}$ and *oxqB*, while NC-1 carried only *qnrS*. Sequence typing showed that, at 5/16 isolates, ST10 was the most prevalent ST, followed by ST181 and ST206 (3/16) and ST101 and ST1638 (2/16).

Conclusions. In summary, we isolated 23 *Enterobacteriaceae* species carrying the polymyxin resistance gene *mcr-1* from seven environmental water samples in China.

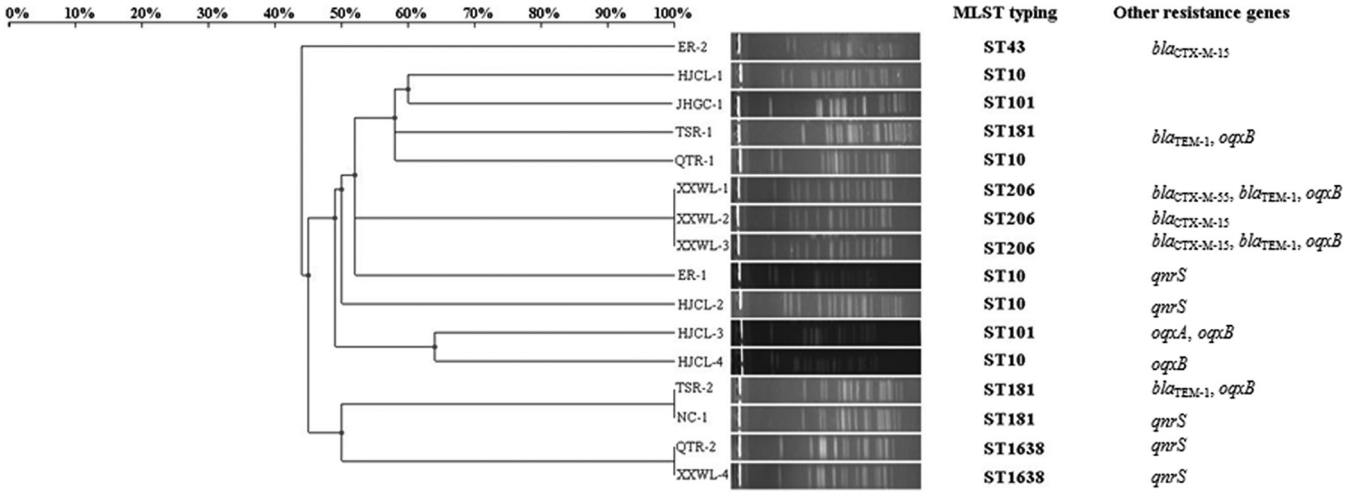

FIG 2 Pulsed-field gel electrophoresis patterns and multilocus sequence typing results for the 16 *mcr-1*-positive *Escherichia coli* isolates. Genomic DNA from the 16 *E. coli* isolates was digested with XbaI restriction endonuclease. ER, East River; HJCL, Huajiachi Lake; JHGC, Jinghang Grand Canal; TSR, Tiesha River; QTR, Qiantang River; XXWL, Xixi Wetland; NC, Nine Creeks.

The enrichment broth culture method performed in this study was useful for isolating *mcr-1*-carrying Gram-negative bacteria from the environment. *mcr-1* was located either on plasmids or in the chromosomal DNA, and plasmid-carried *mcr-1* was successfully transferred between bacteria by conjugation. The responsible use of polymyxins both in agriculture and in the clinical setting is therefore critical to prevent the further spread of colistin resistance.

MATERIALS AND METHODS

Bacterial strains. Water samples were collected from nine distinct aquatic environments (including West Lake, the Qiantang River, the Jinghang Grand Canal, the Xixi Wetland, Huajiachi Lake, Nine Creeks, the Tiesha River, the East River, and the fountain at the Second Affiliated Hospital of Zhejiang University) in Hangzhou, China, in March 2016. The sampling locations represent the main water environments of Hangzhou. We selected five sites at each locality for sample collection, and two 1-liter samples of water were collected from each sampling site using sterile bottles.

Microbial enrichment. Each 1-liter water sample was concentrated by vacuum filtration using filter membranes (<0.2 μm). The membranes were then washed and suspended in 10 ml of sterile 0.45% saline solution. Next, 200-μl aliquots of the suspensions were inoculated into 10 ml of buffered peptone water in 15-ml tubes and were incubated with shaking at 37°C overnight.

Selection of colistin-resistant isolates and detection of *mcr-1* and *mcr-2* by enrichment broth. Each enrichment culture tube was inverted six times to mix the broth and then allowed to stand for 10 min. Aliquot (1 ml)] of the enrichment broths were then transferred into 2-ml tubes. Following centrifugation for 1 min at 10,000 rpm, the supernatants were discarded, and 250 μl of phosphate-buffered saline (PBS) was added to the pellets, which were then boiled for 5 min. The boiled solutions were centrifuged, and 5 μl of the supernatants was used as the template for PCR amplification of *mcr-1* and *mcr-2* as described previously (7, 13). The primers used are listed in Table 2.

Following PCR-based screening, *mcr*-positive tubes were once more inverted six times to mix the remaining broth. After standing for 10 min, 500 μl of broth was transferred to 5 ml of *Enterobacteriaceae* enrichment broth containing 2 μg/ml colistin, which was then incubated at 37°C overnight. Aliquots (10 μl) of the *Enterobacteriaceae* enrichment broth were then spread onto MacConkey agar plates supplemented with 2 μg/ml colistin and the plates were incubated at 37°C overnight. The pure colonies were selected based on their color and morphology, and final isolate identification was conducted using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonik GmbH, Bremen, Germany). The *mcr* genes were further confirmed from the purified colonies by the PCR method as described above.

Molecular typing. MLST was performed as reported previously (24). Seven housekeeping genes were chosen as targets according to the MLST website (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli). Sequence types (ST) were determined based on those in the *E. coli* MLST database.

Pulsed-field gel electrophoresis profiles. The chromosomal genome was prepared in agarose blocks, digested with XbaI and subjected to pulsed-field gel electrophoresis (PFGE) using a Rotaphor system 6.0 instrument (Whatman Biometra, Gottingen, Germany). *Salmonella enterica* serotype Braenderup H9812 was used as a size marker. The pulse times were increased from 1 to 30 s over 24 h at a voltage of 6 V and an angle of 120°. A dendrogram was generated from the homology matrix with a coefficient of 2.0% using the unweighted pair-group method using arithmetic averages ("UPGMA") to

TABLE 2 Primers used for amplifying β-lactamase genes, plasmid-mediated quinolone resistance genes, and colistin resistance genes

Primer name	Sequence (5′–3′)	Reference
β-Lactamase genes		
bla_{TEM}-F	TCGGGGAAATGTGCG	26
bla_{TEM}-R	TGCTTAATCAGTGAGGCACC	
bla_{SHV}-F	GCCTTTATCGGCCTTCACTCAAG	26
bla_{SHV}-F	TTAGCGTTGCCAGTGCTCGATCA	
$bla_{CTX-M-1}$-F	CAGCGCTTTTGCCGTCTAAG	26
$bla_{CTX-M-1}$-R	GGCCCATGGTTAAAAAATCACTGC	
$bla_{CTX-M-2}$-F	CTCAGAGCATTCGCCGCTCA	26
$bla_{CTX-M-2}$-R	CCGCCGCAGCCAGAATATCC	
$bla_{CTX-M-8}$-F	ACTTCAGCCACACGGATTCA	26
$bla_{CTX-M-8}$-R	CGAGTACGTCACGACGACTT	
$bla_{CTX-M-9}$-F	GTTACAGCCCTTCGGCGATGATTC	26
$bla_{CTX-M-9}$-R	GCGCATGGTGACAAAGAGAGTGCAA	
Plasmid-mediated quinolone resistance genes		
qnrA-F	AAGGAAGCCGTATGGATA	27
qnrA-R	AGCTAATCCGGCAGCACTAT	
qnrB-F	CGACCTGAGCGGCACTGAAT	27
qnrB-R	TGAGCAACGATGCCTGGTAG	
qnrC-F	GGGTTGTACATTTATTGAATC	27
qnrC-R	TCCACTTTACGAGGTTCT	
qnrD-F	CGAGATCAATTTACGGGGAATA	27
qnrD-R	AACAAGCTGAAGCGCCTG	
qnrS-F	ACCTTCACCGCTTGCACATT	27
qnrS-R	CCAGTGCTTCGAGAATCAGT	
aac(6′)-*Ib*-F	TGACCTTGCGATGCTCTATG	27
aac(6′)-*Ib*-R	TTAGGCATCACTGCGTGTTC	
qepA-F	CGGCGGCGTGTTGCTGGAGTTCTT	28
qepA-R	CCGACAGGCCCACGACGAGGATGC	
oqxA-F	CTCGGCGCGATGATGCT	29
oqxA-R	CCACTCTTCACGGGAGACGA	
oqxB-F	TTCTCCCCCGGCGGGAAGTAC	29
oqxB-R	CTCGGCCATTTTGGCGCGTA	
Colistin resistance genes		
mcr-1-F	CGGTCAGTCCGTTTGTTC	7
mcr-1-R	CTTGGTCGGTCTGTAGGG	
mcr-2-F	TGTTGCTTGTGCCGATTGGA	13
mcr-2-R	AGATGGTATTGTTGGTTGCTG	

describe the relationships among PFGE profiles. Isolates were considered to belong to the same PFGE group if their Dice similarity index was ≥80%.

Transconjugation and S1-PFGE hybridization. The *mcr-1*-carrying strains isolated in this study were used as donors for transconjugation assays, while rifampin-resistant *E. coli* EC600 served as the recipient. The transconjugants were screened on a medium containing rifampin (600 μg/ml) and colistin (2 μg/ml). Assays were performed as described by Liu et al. (7). Specific PCR amplification was conducted to confirm the presence of *mcr-1* and other resistance genes (such as bla_{TEM}, bla_{CTX-M}, and PMQR genes) in transconjugants. The DNA profiles of representative *mcr-1*-positive isolates, their transconjugants, and untransferable isolates were obtained by S1 nuclease PFGE and hybridization as previously reported by Bai et al. (25).

Antimicrobial susceptibility testing. *In vitro* antimicrobial susceptibility testing was performed to examine the susceptibility of the original isolates and the transconjugants to imipenem (IPM), gentamicin (GEN), cefotaxime (CTX), piperacillin-tazobactam (TZP), cefoperazone/sulbactam (SCF), amikacin (AMK), ticarcillin-clavulanic acid (TIM), cefepime (FEP), ceftazidime (CAZ), ceftriaxone (CRO), and ciprofloxacin (CIP). Assays were performed using the Etest method in accordance with the Clinical and Laboratory Standards Institute guidelines. The MIC of colistin (CST) was determined by broth microdilution. *E. coli* ATCC 25922 was used for quality control. Mueller-Hinton agar and broth (Oxoid, Hampshire, UK) were used for all assays, and MIC results were determined following incubation at 35°C for 18 to 20 h.

PCR amplification and sequence analysis. Primers specific for the β-lactamase genes, including bla_{TEM}, bla_{SHV}, group 1 bla_{CTX-M}, group 2 bla_{CTX-M}, group 8 bla_{CTX-M}, and group 9 bla_{CTX-M}, were used in this study (Table 2) (26). The PMQR genes, including *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *aac*(6′)-*Ib-cr*, *qepA*, *oqxA*, and *oqxB*, were identified as reported previously (27–29) (Table 2). Colonies were boiled to prepare DNA templates for PCR analysis. The reactions were conducted in a T-personal thermal cycler (Whatman Biometra, Gottingen, Germany). PCR products were sequenced using an ABI 3730 sequencer (Applied

Biosystems, Foster City, CA). The sequences were compared with reported sequences from the GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

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